Using various lactic acid bacteria strains during forage conservation towards fermentation, storage, nutritive value and safety improvement

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ABSTRACT

Many research efforts have been devoted to find ways how to produce silages with maximum nutritive value, good fermentation and low mould and yeast counts. While there are different objectives in using silage additives, the main objectives are to improve fermentation and reduce dry matter loss, and to prevent secondary fermentation at feed out time. This paper presents our most significant and recent investigations on forage inoculation before ensiling that have a potential for beneficial application on grassland and ruminant nutrition.

Keywords: Feeding value, Grass and legumes silage, Hygienic quality, Inoculants

Introduction

Milk and beef production in different countries or climatic zones is based on utilization of legumes and grasses or other green plants. Due to a short grazing period in northern countries, grazed grass cannot contribute more than 25-50% of total feed energy intake for cattle. Therefore, the importance of conserved forages, mainly silage, has increased. Moreover, the importance of ensiling has also steeply increased in tropical and sub-tropical regions. Increased demand for milk and meat in tropical and sub-tropical regions required to further increase silage production. There is a great potential for silage making because the yield of tropical plants is three or four time greater than in northern countries. However, acetic acid fermentation often takes place in tropical grass forage due low contents of dry matter and water soluble carbohydrates (Catchpoole and Henzell, 1971), and a high concentration of ethanol may occur in whole crop maize and sugarcane ensiled in the tropics (Pedroso *et al.,* 2005). There have been major advances in the science and practice of silage making in the world over the last 45 years. First of all, silage research has focused on the production and ensiling of grass and legume silages with reference to reduction of dry matter losses and improving fermentation. Later on, researches have dealt with the ensiling and use of silages from a wide range of crops including whole crop maize, whole crop cereal, tropical forages and by-products. Moreover, research on silages included studies concerned with aerobic stability, microbial environment, hygiene, silage intake by animal, animal production, including the effect on animal products, hygiene and safety for humans, and silages technology impact on the environment (Wilkins and Wilkinson, 2015). To this day, we have the knowledge and understanding how microorganisms affect silage quality and understanding of the dynamics of microbial populations at ensiling time. However, we still have a difficult time knowing how significant the new species are to silage preservation (Muck, 2012).

Silage quality and nutrient use efficiency is influenced by a number of factors: such as

crops, ensiling technologies, used machinery and additives for manipulating fermentation processes (Davies *et al.*, 2005). Biological additives were evaluated for their potential to improve silage fermentation and nutritive value (Filya *et al.*, 2007) and for their ability to increase animal productivity (Contreras-Govea *et al.*, 2009). Animal feeding trials (e.g., Weinberg and Muck, 1996) show that some lactic acid bacteria inoculants have improved milk production, increased daily weight gain and improved feed conversion ratio even when the inoculant did not affect silage fermentation compared to ordinary made silage. The Deutsche Landwirtschafts-Gesellscaft Guidelines for the testing silage additives take into account the two main actions which are improving the fermentation process on the one hand and improving aerobic stability on the other hand (Kleinschmit *et al.*, 2005; Weib *et al.*, 2011). The companies producing inoculants expect that new strains and mixtures will be highly competitive and improve silage fermentation by reducing pH and by producing largely lactic acid, compared to spontaneous fermented silage. However, some authors reported that homo-fermentative LAB inoculants did not improve the aerobic stability of silages (Sucu, and Filya, 2006). After opening of the silo, yeast and molds can lead to an increase in pH-value and temperature of the silage as well as to a reduction of free available sugars. Loss of carbon dioxide and temperature increase causes DM losses and reduces the feeding value of silage (Muck, 2012).

Many research efforts have been devoted to find ways how to produce silages with maximum nutritive value, good fermentation and low mould and yeast counts. Numbers of papers on forage species, moisture levels, pack densities, silo sealing materials, as well as silage additives have been published over the last years. This paper reviews the results of few laboratory scale (mini-silo) studies and commercially sized (big scale) studies focused on the effects of the inoculants containing various combinations of novel bacterial strains on fermentation end-products, nutritive value, microbial population and aerobic stability of different silages. The objectives of the reviewed studies were:

Objective 1. *To assess which mixtures of lactic acid bacteria have a greater potential to improve fermentation pattern of the alfalfa, perennial ryegrass and red clover/ryegrass/timothy silage and whether the inoculants have an effect on the extent of deterioration occurring during the exposure to air (laboratory scale).*

An *in vitro* study using mini silos was conducted to observe the effects of silage inoculants on nutrient composition, pH, VFA concentration, DM loss, and aerobic stability. 3 L mini silos were filled to a target density of 0.2 kg DM/L with: alfalfa (3-year-old, first cut, in early-flower stage), perennial ryegrass (2-year-old, first cut, in early-boot stage) and a mixture of red clover:ryegrass:timothy (60:25:15, 2-year-old, first cut, in early bloom stage of red clover). Eight inoculant treatments: Feedthech F10 - *P. acidilactici* 33-11 NCIMB 30085, *P. acidilactici* 33-06 NCIMB 30086, *L. plantarum* LSI NCIMB 30083, *L. plantarum* L-256 NCIMB 30084, *E. faecium* M74 NCIMB11181; Feedthech F18 - *P. acidilactici* 33-11 NCIMB 30085, *P. acidilactici* 33-06 NCIMB 30086, *L. plantarum* LSI NCIMB 30083, *L. plantarum* L- 256 NCIMB 30084, *E. faecium* M74 NCIMB1118, xylanase; Feedthech F22 - *Lc. lactis* SR 3.54 NCIMB30117, *P. acidilactici* 33-11 NCIMB 30085, *P. acidilactici* 33-06 NCIMB 30086, *E. faecium* M74 NCIMB1118, xylanase, sodium benzoate; Feedthech F3000 - *Lc. lactis* SR 3.54 NCIMB30117, *P. acidilactici* 33-11 NCIMB 30085, *P. acidilactici* 33-06 NCIMB 30086 *E. faecium* M74 NCIMB11181, *L. plantarum* MiLab 393 LMG 21296; Sil-All 4 × 4

(ISA) - *E. faecium*, *L. plantarum*, *P. acidilactici*, *L. salivarius*, cellulase, hemicellulase, pentosanase, amylase; Lalsil Dry (LD) - *Lactobacillus buchneri*, *Pediococcoccus acidilactici*, sodium benzoate, beta glucanase; Bonsilage (IBO) - *L. buchneri*, *P. pentosaceus* and Biosil Stabil (BS) - *L. plantarum* DSM 8862, *L. plantarum* DSM 8866, potassium sorbate and one control (CTL- no inoculants) were organized as a randomized complete block design, with five replicates per treatment. Silages were stored for 90 d at 20°C. After 90 days of storage, silages were sampled and analyzed for DM loss and chemical composition (DM, CP, NDF, and pH). Aerobic stability was measured on day 90 of storage by exposing the silages to air and measuring temperature until silages were 3°C above ambient temperature. Mean nutrient composition of herbages (%) was 35.5, 33.2 and 34.3 for dry matter, 22.1, 15.0 and 18.7 for crude protein, 4.2, 12.2 and 9.1 for water soluble carbohydrates and 37.6, 43.5 and 41.3 for neutral detergent fiber for alfalfa, perennial ryegrass and red clover/ryegrass/timothy herbages Mean nutrient composition of silages (%) was 33.8, 32.2 and 32.8 for dry matter, 20.6, 14.7 and 19.0 for crude protein, 0.3, 3.3 and 1.7 for water soluble carbohydrates and 38.0, 44.1 and 42.2 for neutral detergent fiber for alfalfa, perennial ryegrass and red clover/ryegrass/timothy silages. By using inoculants, products of fermentation are shifted in alfalfa, perennial ryegrass and red clover/ryegrass/timothy silages, resulting in significantly more lactic acid concentration and acetic acid concentration and significantly less butyric acid, alcohols and N-NH_3 fraction compared to the silages without additives. Spontaneous fermentation in the control silages produced lower concentrations of fermentation acids, however, used more sugars available in the herbages with less pH drop. This indicates that the addition of inoculants allowed a more rapid production of lactic acid which suppresses the buffering effect of legumes and grasses as suggested by Adesogan and Salawu (2004). The slower decline in pH 3 days after ensiling of spontaneous fermented silage compared to the inoculated silage probably reflected the low epiphytic LAB counts and their less efficient lactic acid production compared to a commercial strain as suggested by Davies *et al.* (2005). Lactate increased in perennial ryegrass for F18 and F22 compared with other inoculants (9.1 v. 6.6; overall mean ± SEM, 6.8 ± 0.57; P<0.01) and red clover/ryegrass/timothy for F10, F22, F3000, and ISA versus other inoculants (5.8 v. 4.3; 4.9 ± 0.2; P<0.01). After 90 days of storage, pH was lower in alfalfa treated with F18, F22, F3000, and SA versus other inoculants (4.88 *vs* 5.02; 5.1 ± 0.01; P<0.01), in perennial ryegrass for F10, F22, F3000, and SA versus other inoculants (4.02 *vs* 4.21; 4.13 ± 0.02; P<0.01), and in red clover/ryegrass/timothy treated with F10, F22, and F3000 compared with other inoculants (4.02 *vs* 4.18; 4.15 ± 0.03; P<0.01). Loss of DM was reduced in perennial ryegrass for F10, F18, F22, F3000, and BO compared with other inoculants (2.9 *vs* 3.8%; 3.5 ± 0.7; P<0.01), and reduced in red clover/ryegrass/timothy for F10, F22, and F3000 compared with other inoculants (4.02 *vs* 4.18; 4.6 ± 0.4; P<0.01). The results demonstrate that every inoculant improved aerobic stability significantly (P<0.05) when compared individually to the uninoculated control. Adding inoculants improved (P<0.05) aerobic stability compared with the control (alfalfa, 229.8 *vs* 98.4; perennial ryegrass, 197.7 *vs* 96.0; red clover/ryegrass/timothy, 167.1 *vs* 80.4). Broberg *et al.* (2007) and Ratanapibulsawat *et al.* (2005) have isolated LAB strains from silages that produce inhibitory activity against a variety of undesirable bacterial species and improve aerobic stability of the silages.

Objective 2. To evaluate the effects of inoculants containing various combinations of novel bacterial strains on the fermentation end-products and aerobic stability of grass silage (laboratory scale).

Forage (70% perennial ryegrass and 30% timothy), wilted to a dry matter (DM) concentration of 265 g/kg was precision chopped and ensiled in 3.0-L silos. The forage was treated with T2 - *Lactobacillus buchneri* CCM 1819/ DSM 22501; T3 - *Lactobacillus plantarum* DSM16568, *Enterococcus faecium* NCIMB 11181/DSM 22502 and *Lactobacillus buchneri* CCM 1819/ DSM 22501; T4 - *Lactobacillus plantarum* DSM16568, *Enterococcus faecium* NCIMB 11181/DSM 22502 and *Lactococcuslactis* DSM 11037; T5 - *Enterococcus faecium* NCIMB 11181/DSM 22502, *Lactococcus lactis* NCIMB 30117 and *Lactobacillus plantarum* DSM16568; T6 - *Lactobacillus plantarum* DSM16568 and *Lactobacillus plantarum* DSM18112. The silages were stored for 90 days at a constant temperature of 20 ^{0}C and after followed by analyses for DM, lactic acid and VFA, ethanol, ammonia-N, number of clostridia spores, yeasts and moulds and an aerobic stability test lasting for 13 days. The aerobic stability of the silages was measured as a number of days reaching a temperature of 3^{o}C above ambient temperature. The quality of inoculated silages was significantly increased compared to that of the untreated, control silage. Products T4, T5 and T6 had significantly higher dry matter concentration and lower DM loss compared with the untreated silage. The T5 silage had the lowest ($P<0.05$) DM loss. All five products resulted in significantly ($P<0.05$) lower pH reduction after 3 d and 90 d of fermentation compared with the untreated silage. Filya *et al.* (2007) concluded that the main effects of silage inoculants were increased production of lactic acid connected with significant reduction of the pH value and minimised DM losses. When compared with untreated silage, increased ($P<0.05$) acetic acid concentration and decreased lactate:acetate ratio were observed only in the T2 silage. The treatments T3-T6 produced higher ($P<0.05$) lactic acid concentration and higher lactate:acetate ratio compared with the untreated and T2 silage. The acetic acid concentration was highest in the T2 silage. The inoculation resulted in lower proteolysis compared to control silages as the inoculated silage had significantly lower concentration of ammonia-N. The inoculated silages also had lower concentrations of ($P<0.05$) alcohols and butyric acid. The LAB blends used in our experiment significantly suppressed yeast and mold growth and was reflected in a lower concentration of alcohols, generally correlated to yeast activity in silage. The highest aerobic stability and lowest yeast and mold counts were found in T2 and T3 silages. The improved stability was related to the lowest pH value after aerobic exposure for 7 days. The aerobic stability of T4-T6 silages was improved by 2.7 d (66 h), the aerobic stability of T3 silages was improved by 5.7 d (138 h) compared to control silages. The aerobic stability of T2 silages was improved drastically. Danner *et al.* (2003) provide evidence for the existence of certain LAB strains with the power to inhibit yeasts and mold growth and to improve aerobic stability.

Objective 3. To evaluate the efficacy of a blend of bacterial strains containing Entrococcus faecium (DSM 22502/NCIMB 11181; Lactococcus lactis (NCIMB 30117 and Lactobacillus plantarum DSM16568 as a microbial inoculant on the chemical composition, fermentation end-products, DM recovery, aerobic stability, and mold development of big bale lucerne silage, and B) To test the zootechnical effect by feeding the inoculated silage as the only forage source to

lactating dairy cows.

The silage inoculant tested in our experiment was a blend of homofermentative lactic acid bacteria strains *Entrococcus faecium* (DSM 22502/NCIMB 11181) *Lactococcus lactis* (NCIMB 30117 *Lactobacillus plantarum* DSM16568. One variety from one field of lucerne (*Medicago sativa*) was used. A homogenous plot of the primary growth of lucerne, at budding stage was mown, wilted to a dry matter concentration of about 35 % and baled into a 1.2 m wide and 1.2 m diametre cilindrical bales. The following additive treatments were applied to the forage in the windrows: Control (T1) – no additive and T2 - inoculated. Five big bales from each treatment chosen at random were core sampled after 90 days of storage for chemical and microbial analyses. The aerobic stability was measured in the laboratory using data loggers that recorded temperature readings from thermocouple wires placed in the core sampled 1000-g silage representative samples aerated in open polystyrene boxes and done in big bales by inserting 70 cm long temperature sensors into the removed of plastic film bales at 2 different points. At the time of removing plastic film and at the end of the aerobic stability test of uncovered big bales, the number of bales with visible surface mould was noted and all visible signs of mould growth on the bale surface were located, numbered and scored using a scale from 0 to 5.

Thirty six multiparous cows were randomly allocated to the control or inoculated silage treatment (of 18 animals each) and treatments were initiated that day. During the experiment, fresh silages were offered *ad libitum*, allowing for at least 10% orts (as-fed basis). No other source of forage than the Lucerne silage was fed to the dairy cows. All cows were also fed a fixed amount (7.13 kg/day as fed) of concentrate and a commercial mineral mixture. The samples of silages from the bales used were taken weekly + the samples of refusals – both were analyzed for nutrients. Milk samples were collected twice daily (a.m. and p.m.) on a weekly basis and were analyzed for fat, protein, lactose and urea content, somatic cells, bacterial contamination, inhibitors and content of *Clostridium perfringens.*

The data of fermentation products, together with the changes in the silage WSC, lactic acid, pH and proportion of ammonia-N during the ensiling period shows that there were clear differences between the treatments in the rate and type of fermentation. Inoculant treatment decreased the pH significantly by 0.54 units ($P<0.05$). There were also more WSC (4.1 g kg^{-1} DM, $P<0.05$) and lactic acid (39.5 g kg^{-1} DM, $P<0.05$), and less alcohols (2.0 g kg^{-1} DM, $P<0.05$) and ammonia N (15.7 g kg N) in the inoculated silage than in the untreated silage after 90 days of ensiling. Regarding other fermentation acids, only 0.9 g kg^{-1} DM butyric acid was detected in the inoculated silage, while 3.9 g kg^{-1} DM was found in the untreated silage. Fermentation losses of the inoculated and untreated silages were 47.1 g/kg DM and 87.6 g/kg DM respectively. Positive outcomes such as higher lactate: acetate ratios, lower ammonia N, decreased DM losses (Henderson, 1993), increased digestibility, improved aerobic stability and enhanced growth performance (Mc Allister *et al.*, 1995) have been reported. In our experiment clostridia was not detected (<1.0 cfu/g) in both silages, therefore, higher ethanol concentration of the untreated silage presumably resulted from the activity of yeasts. Appreciable decrease in the number of yeast (2.07 log cfu/g and molds (2.66 log cfu/g) was detected in the inoculated silage when compared with the untreated silage (1.18 log cfu/g and 1.87 log cfu/g), respectively. Lactobacilli numbers of the inoculated silages increased significantly

($P<0.05$) compared with the control fermentation. At the time of removing plastic film the inoculated bales had no visible surface fungal contamination, when control bales were contaminated with one visible colony each. 18 days after removing plastic inoculated big bales were scored as 1.8, that was 2.3 time lower ($P<0.05$) compared with the untreated big bales. The lower temperature in the inoculated silages relative to the control illustrated the improved aerobic stability afforded by inoculation. The aerobic stability of the inoculated lucerne silage was improved by 180 (7.5 days), compared to the control silages. The cows fed the inoculated silage consumed significantly more dry matter and had a mean milk yield significantly higher than the cows fed the untreated silage. The adding of the inoculant resulted in the lower milk urea nitrogen content compared to the control silage. This indicates that less protein breakdown may have occurred in the inoculated silage (Meeske *et al*., 2000). Weinberg *et al*. (2004) suggested that microbial inoculants may produce a probiotic effect in the rumen, the mechanism of which is unknown. Due to a higher milk fat and milk protein and milk lactose content with the inoculated silage diet energy-corrected milk (ECM), and milk fat, milk protein and milk lactose production was significantly higher compared with the control silage diet. Overall, feed efficiency of dairy cows fed the inoculated silage was significantly greater than that of the dairy cows fed the control silage.

Conclusions

Homo and hetero lactic acid bacteria based inoculants or blends of lactic acid bacteria and other components changed the fermentation profile of grass and legumes silages by decreased pH value, shifted lacate: acetate ratio, protected the silages against proteolysis and butyrate formation, resulted in lower dry matter losses, suppressed yeast and mold growth and improved aerobic stability. *L. buchneri* or blends of hetero-and homo fermentative lactic acid bacteria were more effective in improving aerobic stability. Inoculation reduced visible mould growth on big bales surface.

The improvement in silage fermentation and nutritive value characteristics as a result of inoculation was reflected in dairy cow performance. Feeding inoculated relative to untreated Lucerne big bale silage to dairy cows shows benefits in terms of dry matter and metabolisable energy intake, milk yield and milk safety.

Challenges for the future

Silage production is growing in importance worldwide as the demand of milk and beef production increases, as well as, increases the importance of food safety, which depends on the hygienic quality of forages consumed by animals. Researches and farmers emphasized the importance of the efficient forage conservation technologies that minimize nutrient losses during harvesting, fermentation, storage and aerobic deterioration during feeding out, and improve hygienic quality (safety) of conserved feeds. Therefore, stimulating new research, developing new laboratory procedures to evaluate the value of conserved forages, the application of new technologies and silage additives to silage making will continue to improve. Recent understanding of silage microbiology led to the development of more effective microbial silage additives which must continue by multidisciplinary collaborations. There are many challenges ahead in advancing silages quality, where selection of the grasses and other green plants can play an important role. The environmental temperature at ensiling

may also affect the resulting silage fermentation and may affect the activity of an inoculant bacteria. Consideration of the crop and the way we manipulate both its composition and the natural and added microflora, before and during ensiling, would seem to provide opportunities. A deeper understanding of the role of epiphytic microflora and lactic acid bacteria added, and novel plant breeding criteria would result in mechanisms of silages fermentation control and would improve feeding value and hygienic quality of silages and increase the safety in food chain. A number of recently published papers have reported the results of gas emission from silages. DM losses not only represent a loss of forage nutrients but also indicated the production and emission of volatile organic compounds. That contributes to the greenhouse effect and climate change. Therefore, the identification and quantifying of volatile organic compounds emitted from different silages is important.

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